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Malaria is the most significant of parasitic diseases accounting for at least one million deaths and over 100 million clinical cases each year in endemic tropical areas of the world. The main thrust of our proposed research is to optimize a tricyclic antimalarial agent for safety and efficacy. Selected tricyclics that have been prepared by us are able to inhibit growth of multidrug resistant strains of Plasmodium falciparum by 50% (IC50) at concentrations well below 1 nanomolar. These same compounds are without cytotoxicity toward human bone marrow progenitors until the concentration is increased more than 10,000-fold. We are currently evaluating these optimized tricyclics in a mouse model of malaria in order to assess toxicity and efficacy.

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INTRODUCTION

Malaria is the most significant of parasitic diseases accounting for at least one million deaths and over 100 million clinical cases each year in endemic tropical areas of the world. The main thrust of our proposed research is to investigate the development of a tricyclic antimalarial agent through lead drug optimization procedures involving structure-activity profiling of compounds as selective antimalarial agents and inhibition of hemozoin formation. This proposal also involves a plan to evaluate candidate agents for efficacy in a rodent model of malaria and to optimize the lead compound for in vivo efficacy. Our planned studies are well underway and we are making progress in every facet of the investigation. Prior to this year our studies had focused primarily on the xanthone nucleus as a pharmacophore for tricyclic antimalarial drug design however this year we adopted the structurally similar acridone nucleus as well. As you will see from this report, we have been successful in lead optimization of both xanthones and acridones and have produced compounds that are exquisitely and selectively potent antimalarial agents.

PLANS

The specific and technical objectives of this project have not changed from the original proposal and our research plans remain unaltered. In the next budget cycle we will continue with our efforts to evaluate the safety, cytotoxicity, mutagenicity, and efficacy of tricyclic xanthone and acridone derivatives *in vivo* in an animal model of malaria, and in vitro against a panel of multidrug resistant strains of *P. falciparum*. Mechanistic studies to improve our understanding of molecular basis of the selective antimalarial activity of modified xanthones and acridones will proceed alongside of experiments to monitor the metabolism of test drugs *in vitro* in the presence of microsomal enzymes. All of the information will be assimilated and utilized in an iterative manner with strategic modifications to the structure of our lead compounds to improve *in vivo* efficacy.

BODY OF NARRATIVE

Chemical synthesis and testing of novel xanthone derivatives. We became interested in halogenation of the most active derivatives, C5 and C6. There are multiple explanations for our interest. First, chlorine and fluorine atoms are considered "isosteres" of hydroxy groups in biological systems and we have published data pointing to a higher degree of hydroxylation correlating to improved potency for the xanthones (4, 5). Second, placement of one or more halogens on the xanthone nucleus should diminish the likelihood of biological oxidations at those and other positions around the aromatic core. Third, halogens may facilitate complexation to heme by mesomeric (resonance delocalization of electrons) and/or inductive effects. In the latter case and considering the porphyrin ring current theory (12), we anticipated that halogen attachment to the aromatic xanthone ring at positions 4 and/or 5 would create an electron deficient carbon atom directly above the electron rich ring current of heme – strengthening the interaction by charge-transfer, dipole-dipole, or electrostatic interactions (6-8). For these reasons we pursued and now have successfully synthesized the 4,5-difluoro derivative of C5, 3,6-bis-omega-diethylamino-amyloxy-4,5-difluoro-xanthone: Dr. Rolf Winter, working with graduate student Ms. Rosie Dodean, have succeeded in the multistep synthesis. The synthesis was presented in a poster at the recent Fluorine Chemistry meeting sponsored by the American Chemical Society. Preparation of the compound required synthesis of a few key intermediates including 2,2-difluoro-1,3-cyclohexanedione, 2-fluoro-resorcinoldimethylether and 2,4-dimethoxy-3-fluoroacetophenone. Each of these compounds is a new chemical entity. The availability of the latter two compounds made possible a Friedel-Crafts-type reaction that was used to prepare the desired fluorinated derivative of C5. C5 is one of our lead candidate molecules in the xanthone class and we have published evidence that the drug accumulates in the digestive vacuole of Plasmodium parasites, forms a complex with an incomplete reciprocal dimer of heme, and blocks formation of hemozoin. "4,5-difluoro-C5" was screened for in vitro antimalarial potency in side-by-side tests with C5 and proved to be twice as potent, with an IC50 value of ≈20nM against the chloroquine susceptible D6 strain and the multidrug resistant W2 strain. In the next year, the last year of this contract, we will intend to utilize F^{19}-NMR to study its interaction with heme in solution and in situ in parasitized red blood cells. If successful this would be the first demonstration of a drug-heme complex being formed in viable infected red cells. Of course, we are also planning the test the drug in vivo in mice infected with the rodent parasite, *Plasmodium chabaudi.*

CONVERGENCE OF TRICYCLIC DRUG DEVELOPMENT STRATEGIES: TOWARD DEVELOPMENT OF A HEME COMPLEXING TRICYCLIC ANTIMALARIAL AGENT WITH CHLOROQUINE RESISTANCE REVERSAL ACTIVITY. In considering our drug design project and our commitment to development of a safe and effective tricyclic agent for treatment of malaria, we became interested in merging the functionality of our tricyclic xanthones, functionalized for heme binding, with structural features that impart chloroquine resistance reversal activity into a single tricyclic

system. To merge these two design strategies we needed to switch to the **acridone** system, effectively substituting the xanthone ring oxygen atom for a nitrogen atom. With the acridone system we can attach an R-group (i.e., an alkyl amine) at the N-10 position, a required feature of tricyclic reversal agents (1, 3). During the past year we have synthesized a representative number of acridones and screened them for antimalarial activity and resistance reversal activity and the results combine to suggest that our approach is valid. **Surprisingly, the acridone pharmacophore appears to have a greater intrinsic antimalarial potency as compared to the xanthone nucleus (Table 1).** [The IC_{50} value for xanthone itself is in excess of 50μM.] From the table of data it is clearly shown that an alkoxy group at either the 2 or 3 position is favored for antimalarial activity and combining this feature with a chlorine atom at position 6 yields a compound with very impressive antimalarial activity. 2-Methoxy-6-chloroacridone, prepared by hydrolysis of quinacrine, exhibits IC_{50} values in the low nanomolar range against chloroquine susceptible D6 and the multidrug resistant W2 strain. Synthetic procedures were undertaken to modify 2-methoxy-6-chloro-acridone and 3-methoxy-6-chloroacridone to replace the methoxy group with an alkoxy-tertiary amine of the type used to functionalize the xanthones for binding to heme (and to target the drug to the acidic digestive vacuole). Borrowing from our xanthone project, running in parallel with this work, we plan to synthesize and test side chain derivatives with R-groups containing from 2 to 8 carbon atoms and terminating in a cyclic or acyclic tertiary amine. We will also investigate the importance of the nature and positioning of the halogen atom on the opposite ring for in vitro and in vivo antimalarial activity. [Neutral and anionic substitutions will be effected as well to extend the structure-activity relationships.] Based on our xanthone findings we anticipate that an optimal configuration will exist in the form of 3-ω-pyrrolidinoamyloxy-6-chloro-acridone. [Placement of an N-hydroxy group at N-10 may enhance this molecule's intrinsic antimalarial activity (similar to Floxacrine (10)), but this would preclude the convergence strategy.] We have initiated computational modeling of the haloacridone pharmacophore with Spartan and Gaussian software at a very high level of quantum mechanical theory in an effort to identify key features of our most active compounds. The images are shown in color in Figure 1 and it may be seen that positioning of a chlorine atom or a fluorine atom at the 6th position causes a depletion of electronic charge density (density shown at: -0.02/blue to 0.02/red) in the central portion of the ring system (note the appearance of a "blue hole"). This effect, induced by the halogen atom, may prove to be important for enhanced binding to heme and positioning of the pharmacophore directly over the circulating ring current at the periphery of the porphyrin molecule. In our planned studies we will attempt to draw such correlations by comparing heme affinity with antimalarial potency, in vitro and in vivo.

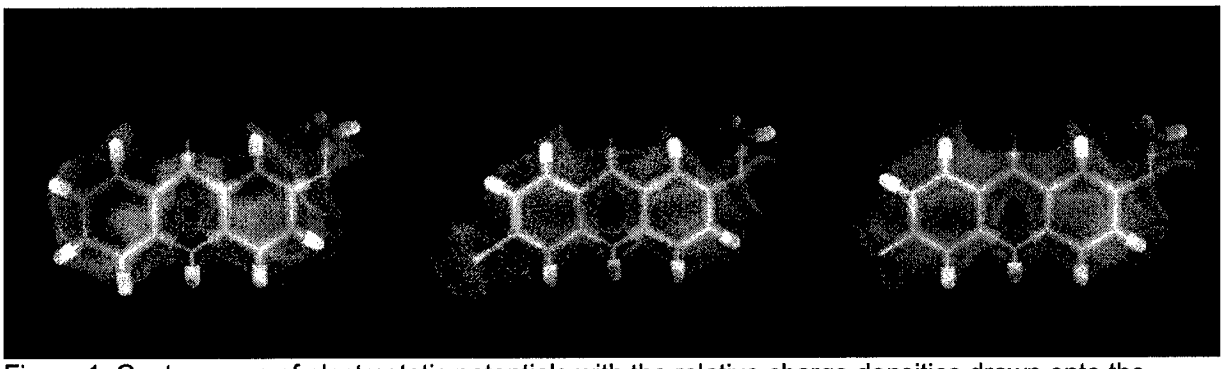
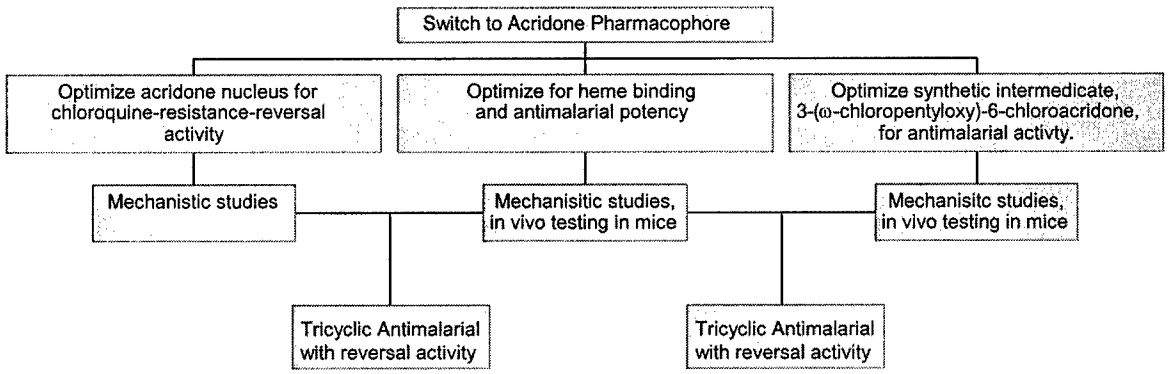


Figure 1. Contour map of electrostatic potentials with the relative charge densities drawn onto the surface. At left is 2-methoxyacridone, in the center is 2-methoxy-6-chloroacridone, and at the far right is 2-methoxy-6-fluoroacridone.

In synthesis of a representative number of additional acridones, we have made a rather remarkable and unpredictable discovery. In order to synthesize the desired substituted aminoalkoxy-halo-acridone we first prepared the corresponding 2- and 3-hydroxy-6-chloroacridone molecules. These compounds provided rather unimpressive antimalarial results. Next, these hydroxy-chloroacridones were converted to the corresponding 2 or 3 (ω-bromo-pentyloxy)-6-chloro-acridone by reaction with dibromopentane. As part of our routine screening procedure all synthetic intermediates are evaluated for intrinsic antimalarial activity by our recently developed fluorescence-based MSF assay (11). As shown in Table 1, both of these compounds exhibit potent

antimalarial activity with IC_{50} values below 100nM. Based on these findings we prepared the same chemical constructs substituting a chlorine atom at the terminus of the pentyloxy unit. These compounds were even more potent with 3-(ω-chloro-pentyloxy)-6-chloroacridone yielding an IC_{50} value of 6.4nM against the D6 strain of *P. falciparum*. Since this molecule has not been functionalized by addition of an alkylamine unit at the terminus of the side chain it is our working hypothesis that this represents a mechanistically distinct and novel class of antimalarial agents. Computer analysis shows that 3-(ω-chloropentyloxy)-6-chloro-acridone has a logP value of ≈4.8 which is not optimal for in vivo efficacy (tests underway presently). Therefore we plan to substitute the aromatic halogen with functionalities that should improve water solubility but retain antiparasitic activity. Among the substitutions that are planned we will evaluate 6-nitro, 6-carboxy, 6-carboxamido, and 6-sulfonamide analogs of the parent compound.

Figure 2. Parallel and Convergent Paths to Design and Optimization of a Tricyclic Antimalarial Agent



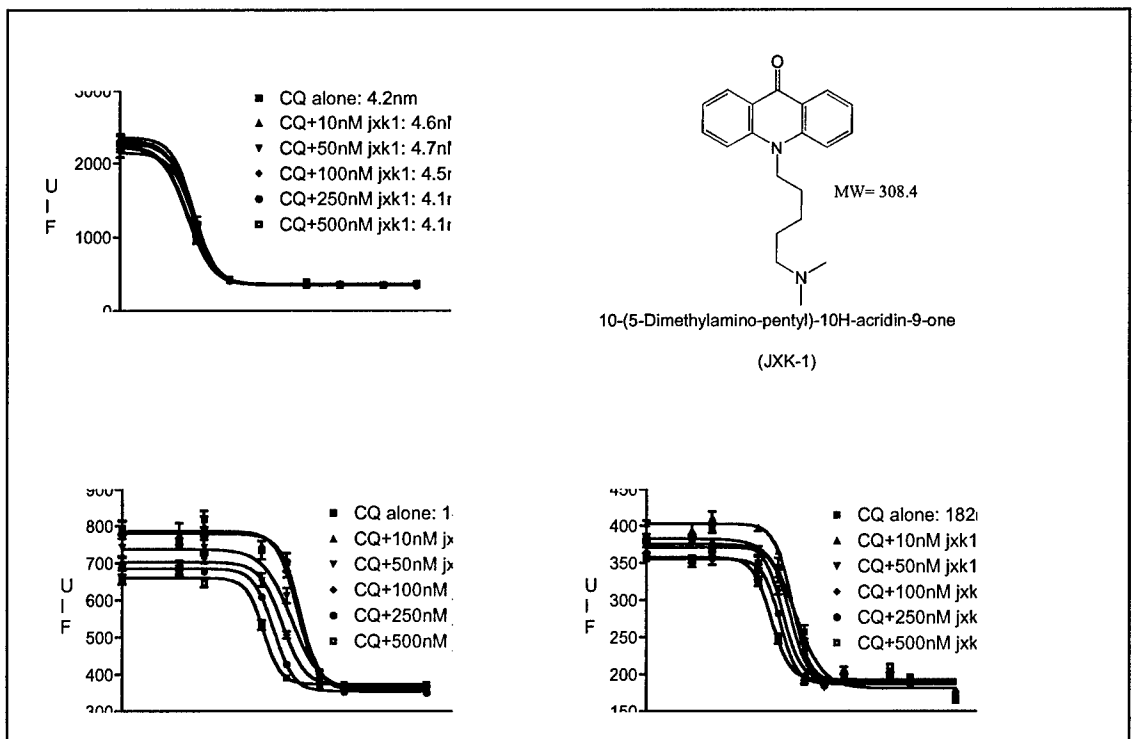


Figure 3. The modified acridone JXK-1 functions as a chloroquine resistance reversal agent against the W2 and 7G8 strains but not the drug susceptible D6 strain.

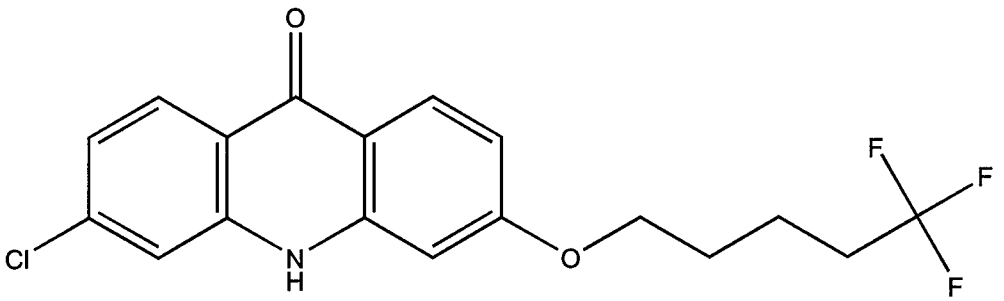
Table 1. In vitro antimalarial activity of selected acridone derivatives against drug susceptible (D6) and multidrug resistant (W2) strains of *Plasmodium falciparum**.

Compound	**D6, IC_{50}, µM**	**W2, IC_{50}, µM**
Acridone	2.9	0.9
2-aminoacridone	>10µM	>10µM
2-methoxyacridone	0.8	0.8
3-methoxyacridone	0.28	0.23
2-hydroxyacridone	9.0	4.4
3-hydroxyacridone	NT	NT
2-methoxy-6-chloroacridone	0.05	0.04
2-hydroxy-6-chloroacridone	0.19	0.45
2-(ω-bromopentyloxy)-6-chloroacridone	0.07	0.17
2-(ω-chloropentyloxy)-6-chloroacridone	0.046	0.042
2-ω-dimethylaminopentyloxy-6-chloroacridone**	NT	NT
3-methoxy-6-chloroacridone	0.10	0.10
3-hydroxy-6-chloroacridone	2.2	8.7
3-(ω-bromopentyloxy)-6-chloroacridone	0.029	0.036
3-(ω-chloropentyloxy)-6-chloroacridone	0.0064	0.022
3-(5,5,5-trifluoromethylpentyloxy-6-chloroacridone (C5-CF3)#	**0.0006**	**0.0006**
3-(4,4,4-trifluoromethylbutyloxy-6-chloroacridone (C4-CF3)	0.001	0.001
3-(6,6,6-trifluoromethylhexyloxy-6-chloroacridone (C6-CF3)	0.0004	0.0004
3-(8,8,8-trifluoromethyloctyloxy-6-chloroacridone (C8-CF3)	0.00017	0.00018
3-(5,5,5-trifluoromethylpentyloxy-6-carboxyacridone**		
3-(5,5,5-trifluoromethylpentyloxy-6-nitroacridone**		
3-ω-dimethylaminoamyloxy-6-chloroacridone**	NT	NT

*Data are the average of at least 3 independent experiments, each performed in triplicate. IC_{50} values were determined by the MSF assay. Results did not vary by more than 10% between the experiments. NT=not tested.

**Synthesis and testing is currently underway. # - Figure shown below.

Note: Compounds of the type shown below may act like atovaquone and/or floxacrine or may not. The compound shown below does not interact synergistically with proguanil, as has been shown for atovaquone. Compd is being modified to enhance solubility Š a multiplicity of approaches are being applied.



3-(5,5,5-trifluoro-pentyloxy)-6-chloro-9-acridone

Additional substitution experiments were considered and some have been reduced to practice. For example, we have prepared a series of acridone derivatives in which the terminal halogen was replaced by a CF_3 moiety. This replacement has been to advantage as the resulting compounds are exquisitely potent and among the most potent antimalarials ever tested in vitro. Prototypical of this group of optimized compounds is

3-(5,5,5-trifluoromethylpentyloxy-6-chloroacridone (C5-CF_3), exhibiting an IC_{50} value of ≈0.6 nanomolar against chloroquine susceptible and multidrug resistant strains of P. falciparum. In considering just how potent these molecules are, one needs to imagine dissolving a single granule of salt in about 20 gallons of water; this would be the concentration of these new chemicals that is needed to completely eradicate the malaria parasites from our laboratory cultures. The new compounds act by a completely different mechanism against strains of the parasite that are resistant to a multiplicity of standard antimalarial drugs. The new compounds are at least 10 times more potent than chloroquine and 100 times more active than quinine, standard drugs used to treat malaria in humans.

IN VITRO CYTOTOXICITY TESTING OF SELECTED TRICYCLIC AGENTS AGAINST HUMAN BONE MARROW PROGENITORS: It is now accepted that morphologically recognizable bone marrow precursor cells are derived from hematopiietic progenitor cells committed to a specific lineage of hematopoietic differentiation. When these progenitors are cultured in vitro under appropriate conditions, they give rise to colonies of mature progeny. Thus, the granulocyte-macrophage progenitor cell (colony forming unit-granulocyte, macrophage; CFU-GM) forms colonies of granulocytes and or macrophages and burst forming units-erythroid (BFU-E) when cultured in semi-solid medium in the presence of human Steel Factor, IL-3, and erythropoietin. Pluripotent stem cells which give rise to committed stem cells such as the CFU-GM and BFU-E undergo self-replication and differentiation into committed stem cells. Gauging the inhibitory effects of candidate antimalarial drugs on the formation of CFU-GM and BFU-E colonies represents an excellent opportunity to assess comparative cytotoxicity against normal mammalian cell proliferation and differentiation and provides evidence of potential for bone marrow suppression. Dr. Grover Bagby, MD, of the Portland VAMC and Director of the Oregon Cancer Center evaluated the inhibitory activity of 3-(5,5,5-trifluoromethylpentyloxy-6-chloroacridone (C5-CF_3) and 3-(6,6,6-trifluoromethylhexyloxy-6-chloroacridone (C6-CF3) against human CFU-GM and BFU-E in vitro. The actual methods that were employed have been published by him previously (2, 9). In summation of his findings, the amount of drug needed to reduce colony formation by 50% (IC_{50}) for both drugs was above 10 μM (micromolar). Taken together, C5-CF3 and C6-CF3 appear to be highly selective and remarkably potent antimalarial compounds with an in vitro therapeutic index (IVTI) of over 10,000-fold for either!

In summary, our ability to draw absolute structure-activity profiles for acridones as antimalarial agents is restricted due to the relatively limited number of acridones that are at hand. However, despite this limitation we have identified a potent antimalarial pharmacophore in the 2-position and 3-position substituted-haloacridones. Even though we have yet to finalize our optimization of these structures for heme affinity and targeting to the food vacuole (by modification of the alkoxy side chain), we have produced drugs that exhibit remarkable activity, which rivals the effectiveness of some of our more potent xanthones. These results suggest that the acridone pharmacophore is superior to the xanthone nucleus (comparative analyses will be carried out in continuance of the work plan) as a platform on which to build a highly active tricyclic antimalarial agent. Our design strategy from this point forward is to proceed along separate paths that will merge within the next year. One path will be focused on structural optimization of the tricyclic haloacridone for antimalarial activity against an extensive panel of *P. falciparum* strains with diverse drug resistance profiles. [Note that we consider analogs of 3-(ω-chloropentyloxy)-6-chloroacridone and 3-(ω-dimethylaminopentyloxy)-6-chloroacridone as mechanistically distinct drug development projects.] Studies will also be conducted in *Plasmodium*-infected mice (IACUC protocols #0404; modified and approved 9/8/04, and #0602, modified and revised earlier this week 2/17/05). A parallel path will focus on incorporation of chemical features into the acridone pharmacophore that are necessary for chloroquine resistance reversal activity (preliminary studies are described below). Once we have separately optimized the acridone structure for both pharmacologic effects (i.e., antimalarial activity and chloroquine resistance reversal activity) we will then attempt to merge both structures into a single compound (Figure 2).

We have also modified acridone to exhibit the reversal phenomenon. Briefly, acridone was converted to 10-N-diethylaminopentyl-acridone by Jane Kelly of our group and screened for reversal activity against three *P. falciparum* strains (7G8 is chloroquine resistant from Brazil). As shown in Figure 3, this congener restores chloroquine sensitivity to resistant strains W2 and 7G8, but is without effect on the susceptible D6 strain as expected. This proof-of-principal experiment demonstrates the versatile nature of the acridone nucleus and supports our hypothesis that we will be able to construct and develop a drug with dual functionality as an antimalarial drug resistance reversal agent. More recent studies have shown that the acridone variant with a 2-carbon chain is without reversal activity, suggesting that there is a correlation between N-10 carbon chain

length and resistance reversal functionality. Drs. Kelly and Winter have recently completed synthesis of the 3, 4, and 6-carbon side chain acridone derivatives, each bearing a terminal diethylamine, and these will be evaluated in due course. It is our intention to synthesize and evaluate the effect of chain length, the nature of the terminal tertiary amine, i.e., cyclic or acyclic, and the effect of ring halogenation on the degree of reversal activity.

Final comments on drug design strategy: As stated earlier, it is impossible to predict whether or not it is feasible for both characteristics to co-exist in the same molecule. For this reason, we plan for our research to move forward along separate paths (1. to develop an antimalarial acridone derivative, and 2) to develop an acridone derivative with resistance reversal properties) and for these to merge, if possible, over next year of the project. Ideally, we will develop an acridone derivative with potent intrinsic antimalarial activity and chloroquine resistance reversal action while exhibiting a pharmacokinetics profile similar to chloroquine itself. This would allow for combination therapy in which the acridone agent and chloroquine could be co-administered on the same schedule. Even if the union concept does not work out, research and development will continue to develop both types of drugs.

KEY RESEARCH ACCOMPLISHMENTS

1. Chemical synthesis and testing of over 30 acridone analogs for structure-activity profiling as antimalarial agents,
2. Chemical synthesis and testing of a difluoro derivative of our lead xanthone, C5.
3. Assessment of lead candidate tricyclic agents for in vitro cytotoxicity against human bone marrow progenitors.
4. Modification of the acridone nucleus in design and optimization of a quinoline-resistance reversal agent,
5. Assessment of off-patent, FDA approved drugs for quinoline-resistance reversal activity; a stereoselective exploitation of a stereo-indifferent target (Smilkstein).
6. Publication of a non-radioisotopic, safe, fluorescence-based method for determination of antimalarial IC_{50} values.

REPORTABLE OUTCOMES

1. **Master's thesis study:** Rosie Dodean from the Dept. of Chemistry, Portland State University, Portland, Oregon. Thesis Title: *"Synthesis of a difluoro xanthone and investigation of its antimalarial mode of action"* Defense of thesis scheduled for Fall 2005.
2. **Publication:** Smilkstein, M, Sriwilaijaroen, N, Kelly, JX, Wilairat, P, and Riscoe, MK, 2003, A simple and inexpensive fluorescence-based technique for high-throughput antimalarial drug screening. Antimicrobial Agents and Chemotherapy 48: 1803-6, 2004
3. **Investigator retraining in tropical disease research:** Dr. Martin J. Smilkstein, MD, has served as an emergency medicine physician at Oregon Health Sciences University for 15 years and head of the Oregon Poison Control Center for ≈10 years of that time. Recently, he joined the organization, Doctors without Borders (MSF), and served in Africa. On return to the Portland area, and after having treated malaria stricken patients in the course of his volunteer effort in Sierra Leone, he decided to pursue research in the field of malaria. He joined our group in January of 2003 and he is actively involved in every aspect of our research endeavors, including this project with PRMRP support.
4. **U. S. Patent No. 6,613,797.** Rolf Winter, Error! Contact not defined., and David J. Hinrichs (Inventors) Xanthone analogs for treating infectious diseases and complexation of heme and porphyrins. Assigned September 2nd, 2003.
5. **Publication:** Winter, R, Kelly, JX, Peyton, D, Hinrichs, DJ, and **Riscoe, MK**, 2004, *Cyclamine analogs of 3,6-bis-alkoxyxanthones with improved antimalarial properties.* Submitted
6. **Poster presentation:** Synthesis and evaluation of N-10-substituted acridone derivatives as chloroquine-resistance reversal agents against Plasmodium falciparum, Jane Kelly, Patricia Pauletti, Martin Smilkstein, Vanderlan da Silva Bolzani, Rolf Winter, and Mike Riscoe, 2004, Annual Meeting of the American Society of Tropical Medicine and Hygiene, Miami, FL.
7. **Poster presentation:** Reversing chloroquine resistance: A stereo-selective approach to a stereo-indifferent target, Martin Smilkstein and Mike Riscoe, 2004, Annual Meeting of the American Society of Tropical Medicine and Hygiene, Miami, FL.

8. **Presentation:** A comparison of fluorescence-based methods for in vitro antimalarial drug testing, Martin Smilkstein, Jane Kelly, and Mike Riscoe, 2004. Annual Meeting of the American Society of Tropical Medicine and Hygiene, Miami, FL.

CONCLUSIONS

The broad and specific aims of this project have not changed from the original proposal and our research plans remain unaltered. In the next budget cycle we will continue with our efforts to evaluate the safety, mutagenicity, and efficacy of xanthone and acridone derivatives in vivo in an animal model of malaria, and in vitro in *P. falciparum*-infected erythrocytes. Mechanistic studies to improve our understanding of molecular basis of the selective antimalarial activity of functionalized derivatives will proceed alongside of experiments to monitor the metabolism of test drugs in vitro in the presence of microsomal enzymes. All of the information will be assimilated and utilized in an iterative manner with strategic modifications to our lead compounds to improve in vivo efficacy.

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